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Invited Review

Chronic granulomatous disease

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Abstract

Introduction: Chronic granulomatous disease (CGD) is a primary immunodeficiency characterized by recurrent, life-threatening bacterial and fungal infections of the skin, the airways, the lymph nodes, the liver, the brain and the bones. Frequently found pathogens are *Staphylococcus aureus*, *Aspergillus* species, *Klebsiella* species, *Burkholderia cepacia, Serratia marcescens* and *Salmonella* species.

Sources of data: CGD is a rare (~1:250 000 individuals) disease caused by mutations in any one of the five components of the NADPH oxidase in phagocytic leucocytes. This enzyme generates superoxide and is essential for intracellular killing of pathogens by phagocytes.

Areas of agreement: CGD patients suffer not only from life-threatening infections, but also from excessive inflammatory reactions.

Areas of controversy: Neither the cause of these inflammatory reactions nor the way to treat them is clear.

Areas timely for developing research: Patient selection for and timing of bone marrow transplantation along with gene therapy.

Key words: chronic granulomatous disease, NADPH oxidase, hyperinflammation, autophagy, IL-1β, autoreactive T cells

Introduction

Clinical symptoms

Patients with chronic granulomatous disease (CGD) suffer from a variety of recurrent bacterial and fungal infections.[1,2] These infections occur most commonly in organs in contact with the outside world—the lungs, gastrointestinal tract and skin, as well as in the lymph nodes that drain these structures. Because of both contiguous (by contact) and haematogenous (via the blood) spread of infection, a wide range of other organs can be affected, most notably the liver, bones, kidneys and brain. In approximately two-thirds of patients, the first symptoms

of CGD appear during the first year of life in the form of infections, dermatitis (sometimes seen at birth), gastrointestinal complications (obstruction or intermittent bloody diarrhea due to colitis) and a failure to thrive. The clinical picture can be quite variable, with some infants suffering from several of these complications, whereas others appear far less ill. In some cases, the presenting symptoms of CGD can be mistaken for pyloric stenosis, food or milk allergy, or iron-deficiency anemia.

Pneumonia is the most common type of infection encountered in CGD in all age groups and is typically caused by *Staphylococcus aureus*, *Aspergillus* species, *Burkholderia cepacia* and enteric gram-negative bacteria. *Aspergillus* and other fungal infections of the lung also pose difficult challenges, because they typically require prolonged treatment (3–6 months). Cutaneous abscesses and lymphadenitis represent the next most common types of infection in CGD and are typically caused by *S. aureus*, followed by various gram-negative organisms, including *B. cepacia* complex and *Serratia marcescens*. Recurrent impetigo, frequently in the perinasal area and caused by *S. aureus*, usually requires prolonged courses of oral and topical antibiotics to clear. Hepatic (and perihepatic) abscesses are also quite common in CGD and are typically caused by *S. aureus*. Patients usually present with fever, malaise and weight loss. Osteomyelitis is another important infection in CGD and can arise from haematogenous spread of organisms (*S. aureus*, *Salmonella* spp., *S. marcescens*) or contiguous invasion of bone, typically seen with non-*fumigatus Aspergillus* pneumonia, such as *Aspergillus nidulans* spreading to the ribs or vertebral bodies. Perirectal abscesses are also common in CGD patients and once formed can persist for years despite aggressive antimicrobial therapy and fastidious local care. Other frequently encountered microbial agents are *Escherichia coli* species, *Listeria* species, *Klebsiella* species, *Nocardia* and *Candida* species.

Not all encounters with microorganisms in CGD result in overt pyogenic infections, as stalemates may develop between the pathogen and the patient's leucocytes. In these cases, chronic inflammatory cell reactions consisting of lymphocytes and histiocytes develop, which can then organize to form granulomas, one of the hallmarks of CGD and causing various clinical symptoms of obstruction (such as antral narrowing of the stomach, delayed gastric emptying, emesis, dysphagia, weight loss, biliary tract or gastrointestinal obstruction, bronchial obstruction, urinary bladder obstruction or ureteropelvic obstruction with kidney dysfunction).

Another important type of chronic inflammation in CGD is a form of inflammatory bowel disease that closely resembles Crohn's disease and affects a substantial fraction of CGD patients.[3,4] While the colon is typically involved, the ileum and other parts of the gastrointestinal tract can also be affected. The symptoms can range from mild diarrhea to a debilitating syndrome of bloody diarrhea and malabsorption that can even necessitate colectomy. Other types of chronic inflammation include non-infectious arthritis, gingivitis, chorioretinitis or uveitis, glomerulonephritis and—rarely—destructive white matter lesions in the brain, discoid or even systemic lupus erythematosus.[5,6]

CGD is a rare disease, occurring about once in every 250 000 individuals. CGD patients usually manifest their symptoms at an early age, in the first 2 years of life. However, due to the diverse genetic causes of the disease (see below), some patients may also present later in life. Most CGD patients (~80%) are male, because the main cause of the disease is a mutation in an X-chromosome-linked gene. However, defects in autosomal genes may also underly the disease and cause CGD in both males and females.

Killing of pathogens

CGD is caused by the failure of the patients' phagocytic leucocytes to kill a wide variety of pathogens. This is due to a defect of these phagocytes to produce reactive oxygen species (ROS), which are needed for the killing process. In normal phagocytes, these ROS are generated by an enzyme called phagocyte NADPH oxidase. This enzyme is composed of five subunits, two of which are in resting phagocytes localized in the plasma membrane and three in the cytosol (Table 1). The two membrane-bound subunits are a transmembrane glycoprotein (gp) with a

Table 1 Properties of the NADPH oxidase components and mutations in CGD patients

Component	gp91^{phox}	p22^{phox}	p47^{phox}	p67^{phox}	p40^{phox}
Synonyms	β-Chain heavy chain Nox-2	α-Chain light chain	NCF-1	NCF-2	NCF-4
Amino acids	570	195	390	526	339
Molecular weight (kDa)					
Predicted	65.0	20.9	44.6	60.9	39.0
By PAGE	70–91	22	47	67	40
mRNA(kb)	4.7	0.8	1.4	2.4	1.4
Gene locus	CYBB Xp21.1	CYBA 16q24	NCF1 7q11.23	NCF2 1q25	NCF4 22q13.1
OMIM	*300 481	*608 508	*608 512	*608 515	*601 488
Exons/span	13/30 kb	6/8.5 kb	16/40 kb	11/15 kb	10/18 kb
Cellular location in resting neutrophils	Specific granule membrane; plasma membrane	Specific granule membrane; plasma membrane	Cytosol; cytoskeleton	Cytosol; cytoskeleton	Cytosol; cytoskeleton
GenBank Accession No.	NM_000397	M21186, J03774	M25665, M26193	M32011	U50720-U50729
Mutations in CGD	Large deletions (Xb^0), small deletions (Xb^0, Xb^-), insertions (Xb^0), nonsense mutations (Xb^0), missense mutations (Xb^0, Xb^-, Xb^+) splice site mutations (Xb^0, Xb^-), promoter mutations (Xb^-)	Large deletions, small deletions, insertions, nonsense mutations, missense mutations, splice site mutations (all leading to $A22^0$, except one missense mutation $A22^-$)	Unequal cross-over with pseudogenes, large deletions, small deletions, nonsense mutations, missense mutations, splice site mutations (all leading to $A47^0$)	Large deletions ($A67^0$), small deletions ($A67^0$, $A67^-$), insertions ($A67^0$), nonsense mutations ($A67^0$), missense mutations ($A67^0$, $A67^-$), splice site mutations ($A67^0$, $A67^-$)	One patient with 10-bp insertion and missense mutation ($A40^-$). Several other patients unreported.
Disease OMIM	#306 400	#233 690	#233 700	#233 710	#613 960

CYBA, cytochrome-*b* α; *CYBB*, cytochrome-*b* β; *NCF*, neutrophil cytosol factor; PAGE, polyacrylamide gel electrophoresis; Xb^0, X-linked (mutation in *CYBB*) without gp91^{phox} protein expression and without NADPH oxidase activity; Xb^-, X-linked (mutation in *CYBB*) with diminished gp91^{phox} protein expression and diminished NADPH oxidase activity; Xb^+, X-linked (mutation in *CYBB*) with normal gp91^{phox} protein expression but without NADPH oxidase activity; A22, autosomal with mutations in p22^{phox} gene *CYBA* (for superscript, see Xb); A47, autosomal with mutations in p47^{phox} gene *NCF1* (for superscript, see Xb); A67, autosomal with mutations in p67^{phox} gene *NCF2* (for superscript, see Xb); A40, autosomal with mutations in p40^{phox} gene *NCF4* (for superscript, see Xb). * is for gene OMIM, #is for disease OMIM.

molecular mass of 91 kD, called gp91^{phox} (phox for phagocyte oxidase), also known as NOX2 (neutrophil oxidase-2), and another transmembrane protein with a molecular mass of 22 kD, called p22^{phox}. These two proteins form a heterodimer and in phagocytes are dependent on each other's presence for maturation and stable expression. This heterodimer is called cytochrome b_{558}, because gp91^{phox} contains two haem groups with an absorbance peak at 558 nm. The three cytosolic subunits (p40^{phox}, p47^{phox} and p67^{phox}) form a heterotrimer that translocates to cytochrome b_{558} upon cell activation (e.g. by binding of microorganisms or chemotactic factors to membrane receptors). As a result, the conformation of gp91^{phox} is slightly changed, which enables NADPH in the cytosol to bind and donate electrons to this protein. These electrons are then transported within gp91^{phox} to molecular oxygen on the apical side of the membrane. In this way, superoxide—the one-electron reduction product of oxygen—is generated within the phagosome that engulfs the ingested micro-organism or on the outside of the phagocyte[2] (Fig. 1). From superoxide, other ROS, such as hydrogen peroxide, can be generated.

These compounds in themselves are not able to efficiently kill microorganisms, but they can give rise to other, very reactive compounds, such as hypochlorous acid (HOCl), hydroxyl radicals (•OH) and secondary amines (R_2NH), which are more toxic. Moreover, phagocytes also produce the reactive nitrogen species (RNS) nitric oxide (NO•) by means of nitric oxide synthase (NOS). This enzyme is

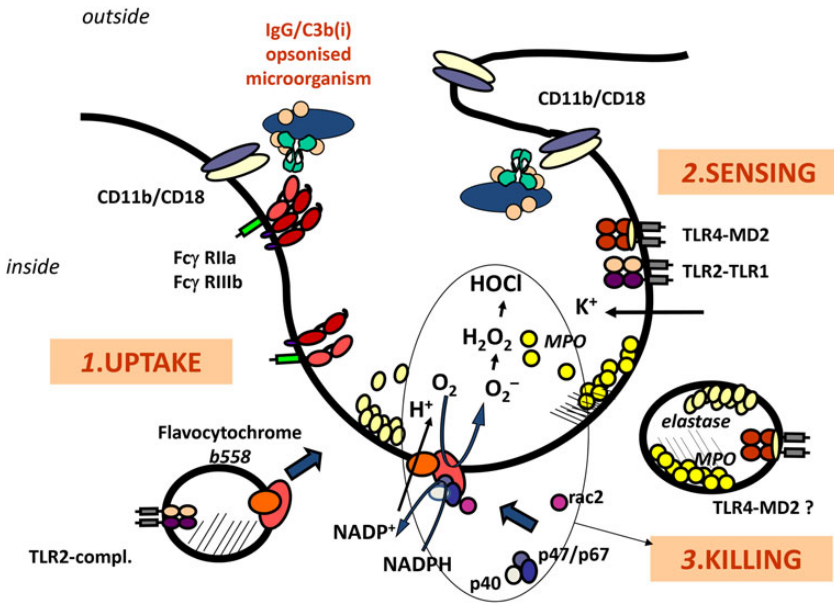

Fig. 1 Phagocytosis and killing of microorganisms by phagocytic leukocytes. Top: A micro-organism, opsonized with antibodies and complement fragments, binds to receptors on the leukocyte surface, such as Fc-gamma receptors and CR3 (CD11b/CD18). This starts the uptake (phagocytosis) of the micro-organism by the phagocyte: pseudopods of the phagocyte fold around the micro-organism and engulf it in a vacuole: the phagosome. At the same time, TLR receptors bind to various microbial ligands (sensing). Subsequently, the NADPH oxidase enzyme complex is assembled from flavocytochrome b_{558} (in the membrane of specific granules) and the cytosolic components p40^{phox}, p47^{phox} and p67^{phox}. This enzyme then starts to produce various forms of ROS in the phagosome. At the same time, azurophil granules, with their content of proteases (such as elastase) and myeloperoxidase (MPO), fuse with the phagosome and deliver these enzymes in the neighborhood of the microorganisms. The phagosome closes during these last steps and killing of the microbes is completed.

present in cells as three isoforms. Most relevant to phagocyte-microorganism interactions is the inducible isoform iNOS, which is synthesized especially in macrophages after binding of microorganisms to microbial pattern recognition receptors on the surface of these cells together with signalling from pro-inflammatory cytokines such as interferons, interleukin-1β (IL-1β) and tumour-necrosis factor-α (TNFα). Due to this delay in production, nitric oxide and its derivatives nitrite (NO^-), nitrosothiol (RSNO), sulphenic acid (RSOH) and nitrogen dioxide ($\bullet NO_2$) are antimicrobial effector molecules that play a sustained role in limiting residual bacterial replication, after the early host response to infection mediated by the ROS produced by the phagocyte NADPH oxidase (reviewed in Ref. 7).

ROS and RNS can act by themselves but can also interact to give rise to other microbicidal intermediates, such as peroxynitrite ($ONOO^-$), with additional reactivity. Moreover, NADPH oxidase activation also leads to potassium ion influx in the phagosome, which helps liberating proteolytic enzymes from the matrix of the granules that fuse with the phagosome after phagocytosis of microbes by the leucocytes.[8] Together, the reactive compounds and the enzymes form a highly toxic mixture that will kill almost all pathogens. Therefore, although CGD patients have a defect only in ROS production, their failure to efficiently kill certain pathogenic microbes may be a direct as well as an indirect effect of this deficiency.

Diagnosis of CGD

At the cellular level, the diagnosis of CGD can be made by measuring the capacity of the phagocytic leucocytes to produce superoxide or hydrogen peroxide.[9] Usually, neutrophilic granulocytes are used for that purpose. These cells are purified from fresh EDTA or heparin blood and activated by fluid or particulate agents. Well-known fluid stimuli are phorbol-myristate acetate (PMA) and the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP). PMA activates intracellular protein kinase C, which phosphorylates $p47^{phox}$ and in this way starts the assembly of the NADPH oxidase complex and the start of the enzymatic activity. fMLP binds to a receptor on the surface of the neutrophils, which leads to NADPH oxidase activation if the cells are pre-activated by, e.g., platelet-activating factor (PAF), TNFα or lipopolysaccharide (LPS). fMLP and (usually) PAF are therefore used in combination to start the enzyme activity. Several particulate stimuli can also be used, such as zymosan (lipid-extracted *S. cerevisiae* membranes), living or heat-killed yeasts (*S. cerevisiae, C. albicans*) and bacteria such as *E. coli* or *S. aureus*. For adequate oxidase activation, these microorganisms are usually opsonized by pre-incubating them in human serum, which leads to coating with antibodies and complement components. Non-opsonized microorganisms bind to specific receptors on the neutrophils such as dectin, Toll-like receptors or complement receptor 3 (CR3), whereas opsonized particles bind to Fc-gamma receptors (for IgG) or CR3 (for the iC3b fragment). This receptor binding again starts a train of intracellular reactions that cumulate in NADPH oxidase activation.

Well-known assays to measure NADPH oxidase activity are the cytochrome *c* reduction assay and the nitro-blue tetrazolium (NBT) slide assay, which both measure superoxide.[9] Cytochrome *c* obtains a different light absorbance spectrum when it is reduced, and this can be measured spectrophotometrically. NBT is also reduced by superoxide and then forms dark blue formazan precipitates in the cells; this can be measured on a per-cell basis by microscopic observation. Water-soluble derivatives of NBT, such as WST-1, also exist. Detailed protocols for these assays can be found in Ref. 10. Both assays are sufficiently sensitive and specific for CGD diagnosis, but rather laborious. The tetrazolium assays are useful when women suspected of being carriers for the X-chromosome-linked subtype of CGD have to be diagnosed: these women have a mixture of neutrophils with intact NADPH oxidase activity and neutrophils with deficient NADPH oxidase (due to random inactivation of one X chromosome in each cell) and therefore display a mosaic of formazan-positive and -negative cells.

Other assays are employed to measure hydrogen peroxide in this context.[9] The best known are the dihydrorhodamine-1,2,3 (DHR) assay and the

Amplex Red assay. The first is based on the oxidation of DHR to rhodamine-1,2,3 by H_2O_2 in the presence of a peroxidase. This leads to a change in fluorescence that can be measured by flow cytometry (FACS) analysis. It is again an assay that measures the NADPH oxidase activity in each separate cell and can thus again be used to detect X-CGD carriership. A pitfall in this assay is the need for peroxidase activity. As such, myeloperoxidase, present in large amounts in neutrophils, fulfils this role. However, in case of myeloperoxidase deficiency, the DHR test will be negative, which may lead to a false-positive diagnosis of CGD. The Amplex Red assay is based on the oxidation of this compound to resorufin in the extracellular medium, again only in the presence of a peroxidase. For this purpose, horse-radish peroxidase is usually added to the cells. The rate of resorufin formation can be followed by the change in fluorescence in a plate reader. We recently realized that the Amplex Red assay overestimates low rates of H_2O_2 generation, possibly because H_2O_2 breakdown by catalase in the cells is minimal under these conditions but much more pronounced at higher H_2O_2 concentrations. Therefore, we prefer the DHR assay as a very sensitive and specific assay that reliably detects all NADPH oxidase deficiencies in neutrophils.

Thus, when a patient is suspected of suffering from CGD on the basis of the clinical symptoms, definite proof can only be obtained with one of the above-described cellular assays. To find the mutation that causes all these problems is an essential next step, to be able to offer genetic advise, perform prenatal diagnosis and eventually to choose a suitable bone marrow donor or perform gene therapy.

Genetics

The genes of the five NADPH oxidase components are *CYBB* (located on the X chromosome) encoding $gp91^{phox}$, and the autosomal genes *CYBA* encoding $p22^{phox}$, *NCF1* encoding $p47^{phox}$, *NCF2* encoding $p67^{phox}$ and *NCF4* encoding $p40^{phox}$ (Table 1). About 70% of the CGD patients have a mutation in *CYBB* (most of them hemizygous males, but a few heterozygous females with skewed expression of their mutation are also known). The remainder of the patients have a mutation in *NCF1* (~20%), in *CYBA* (~5%) or in *NCF2* (~5%). Only one patient has been reported in detail with a mutation in *NCF4*. A loss-of-function mutation or a hypomorphic reduction-in-function mutation in any of these five genes can cause CGD. If the mutation leaves some residual NADPH oxidase activity intact, the clinical expression of the disease is attenuated[11] and the chance of survival of the patient is higher[12] than in case of total oxidase deficiency. This depends on the gene mutated, the type of mutation and on the position of the mutation within the gene. In general, mutations in *NCF1* lead to a milder form of CGD (later presentation, milder clinical expression, better chance of survival) than mutations in any of the other genes.[2,11]

Until recently, cellular investigations were necessary before adequate genetic analysis was possible. To make a choice which gene to analyze, one needed to know the expression of the oxidase component proteins and the indications of a possible X-linked nature of the disease in the family.[9] Nowadays, small gene panels, sequenced on benchtop next-generation sequencing platforms, are used that can detect mutations in multiple genes in one run.[13] Therefore, we now routinely analyze exons and exon–intron boundaries of all CGD genes, including the glucose-6-phosphate dehydrogenase (G6PD) and Rac2 genes.[2,9] G6PD deficiency in severe form may lead to insufficient NADPH formation in leucocytes, thus hampering NADPH oxidase activity.[14] Rac2 (Ras-related C3 botulinum toxin substrate 2) is a small GTPase that is involved in the signal transduction from surface receptors to the NADPH oxidase, and when mutated may lead to CGD-like symptoms.[15] If no mutations are found in any of these genes, it may be that missplicing of mRNA occurs due to deep intronic mutations. Therefore, mRNA size and sequence should then be analyzed. Also for *NCF1*, it is sometimes necessary to analyze the mRNA because analysis of genomic DNA is difficult owing to the presence of two pseudogenes very homologous to *NCF1*.

Treatment

Treatment of CGD should start at the earliest opportunity. This involves determination of the exact complicating infections and selection of the most appropriate antibiotic or anti-fungal therapy. Early and aggressive use of parenteral antibiotics is essential in preventing further spread of the infection. It is usually necessary to begin antibiotic therapy empirically before culture results are available; in these cases, the antibiotics should be chosen to provide strong coverage for *S. aureus* and gram-negative bacteria, including *B. cepacia* complex (e.g. a combination of nafcillin and ceftazidime or a carbapenem; most aminoglycosides are typically ineffective against *Burkholderia*). If the infection fails to respond within 24–48 h, then more aggressive diagnostic procedures should be instituted to identify the responsible microorganism. Additional empirical changes in antibiotic coverage may be warranted, such as adding high-dose intravenous trimethoprim-sulfamethoxazole to cover ceftazidime-resistant *B. cepacia* and *Nocardia*.[2]

If fungus is identified or strongly suspected, antifungal therapy should be started even before the diagnosis is confirmed. *Aspergillus* infections of the lungs and bones are the most common fungal infections and often require prolonged treatment. In general, the newer triazole antifungals are better tolerated and have better activity than amphotericin derivatives. Most patients are assumed to require prophylactic treatment. New triazole antifungals for intravenous and oral use, itraconazole, voriconazole and posaconazole, have already shown great benefit in patients with refractory fungal infections and are the preferred agents in some centres for the empiric treatment of all filamentous mold infections. The echinocandin antifungals (caspofungin, micafungin, anidulafungin) can be used for the treatment of refractory *Aspergillus* infections in patients unresponsive to or unable to receive azoles or lipid-formulated amphotericin B. Certain fungal infections may progress under appropriate therapy in CGD, especially with some of the newly characterized fungi that are morphologically similar to *A. fumigatus* but have distinct and aggressive behaviors in CGD patients. Early treatment with additional intravenous antifungals must be considered.[2]

Surgical drainage (and sometimes excision) of infected lymph nodes and abscesses involving the liver, skin, rectum, kidney and brain is often necessary for healing, particularly for the visceral abscesses. Daily prophylaxis with trimethoprim-sulfamethoxazole or dicloxacillin and with the antifungal medication itraconazole is recommended during infection-free periods. Also prevention of infections through immunizations and at all times avoidance of certain sources of pathogens is recommended. Use of prophylactic recombinant human interferon-γ (rhIFN-γ) induces a 70% reduction in the risk of developing a serious infection,[16] but this concept has been challenged.[17] Suppression of inflammation with blocking agents against TNFα is successful in treating patients with severe colitis but associated with significant infectious complications.[18] Obstructive symptoms should be treated with oral corticosteroids.[19,20]

One of the main reasons for making a rapid diagnosis of the severe forms of CGD is that such patients may be cured with a haematopoietic stem cell transplantation (HSCT).[21–23] This is now successful in over 90% of the patients, but still carries the risk of patients succumbing to overwhelming infections when these are smouldering or overt during the immunosuppressed period of HSCT. Since the success rate is higher when the patients are young and in a disease-free condition, the choice is between transplanting a child that does rather well and waiting until the symptoms are worse, with a less favourable prognosis.

For patients without an HLA-compatible donor, gene therapy would be a good alternative to HSCT. Conventional gene therapy trials for CGD have been based on transfer of a therapeutic transgene into autologous haematopoietic stem and progenitor cells (HSPCs) via retroviral vectors that semi-randomly integrate in the genome. Although this initially led to clinical improvement in the patients, methylation of the viral promoter has resulted in transgene silencing over time and therefore loss of therapeutic benefit.[24,25] Furthermore, insertional activation of several proto-oncogenes led to clonal dominance of corrected cells, eventually leading to myelodysplasia.[25]

New attempts have been directed at gene correction through introducing site-specific double-strand DNA breaks by engineered homing endonucleases, such as zinc-finger nucleases (ZFN)[26–28] or transcription activator-like effector nucleases (TALENs),[29] followed by homologous recombination of the mutated gene region with a similar exogenous wild-type cDNA source through endogenous DNA repair mechanisms. The introduction of site-specific double-strand DNA breaks greatly enhances correction efficiency and carries the advantage of gene correction *in situ*, still under the control of the gene's own cell-specific promoters. However, for gene correction of CGD, this may not be the method of choice, given that many CGD patients suffer from disease caused by unique mutations. This would imply engineering ZFNs or TALENs for each patient separately. Instead, targeting specific so-called safe harbors in the genome whose disruption does not lead to deleterious consequences such as insertional mutagenesis may be an attractive alternative. One such locus, the AAVS1 locus (the common integration site of the adeno-associated virus 2), has been used for lentiviral transduction in a myeloid-specific cassette of induced pluripotent stem (iPS) cells from CGD patients.[28,30,31] iPS cells are epigenetically reprogrammed somatic cells with a high reproduction capacity that can be induced to differentiate into all possible somatic cells, including myeloid cells. The results were encouraging, in that the corrected iPS clones did not show signs of mutations at the top 10 predicted off-target sites of the nucleases used and after differentiation showed strongly upregulated mRNA for the corrected CGD genes and restored ROS production. TALEN-mediated correction was less cytotoxic for the iPS clones than ZFN-mediated correction.[31] However, this targeting of the AAVS1 locus of course lacks the attractive prospect of gene correction *in situ*. Finally, a third way of gene correction has also been tried with CGD iPS cells. This is the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 nuclease system, which does not work with a nuclease (Cas9) that is guided by proteins binding to a specific site in the DNA, such as ZFNs or TALENs, but by a short RNA stretch that fits to the gene at the site to be corrected.[32] Here too, iPS clones were obtained with a corrected CGD gene, in this case *CYBB*, and these clones were successfully differentiated into monocytes and macrophages with restored $gp91^{phox}$ mRNA and ROS production. Since it is much simpler to construct site-specific RNA than protein, the CRISPR-Cas9 technique is a promising tool for patient-specific gene correction. At present, it is not yet possible to apply this technique to HPSCs for permanent correction of sufficient phagocyte precursors in the bone marrow. Limitations are efficiency of gene editing in HSPCs and maintaining the bone marrow-reconstituting potential of these fragile cells throughout the whole procedure of transfection, expansion and selection.

Hyperinflammation

Recent research has concentrated on the origin of the hyperinflammatory state of CGD patients. Two different causes have been proposed, which are not necessarily mutually exclusive. These are the involvement of autophagy in the microbial killing process and the prevention of excessive T-cell activation by phagocytes.

Autophagy

Autophagy is the process in which cytoplasmic contents within a cell are sequestered within a double-membraned vacuole that subsequently fuses with lysosomes, resulting in degradation of the contents.[33] In this way, a cell rids itself of unwanted constituents and recycles these to maintain macromolecular synthesis during stressful conditions. A hallmark event in the autophagic process is the reversible conjugation of the Atg (autophagy-related) 8 family of proteins to the autophagosomal membrane. In mammals, LC3 (light chain 3) is the best known member of this Atg8 family. All Atg8 members serve as substrates for the Atg4 family of cysteine proteases, leading to cleavage near the C-terminus, downstream of a conserved glycine. This cleavage allows the conjugation of the Atg8 proteins to phosphatidylethanolamine (PE) through the exposed glycine (Fig. 2). The lipidated Atg8 proteins associate with the autophagosome and facilitate fusion with lysosomes. Thereafter, the lipidated Atg8 proteins

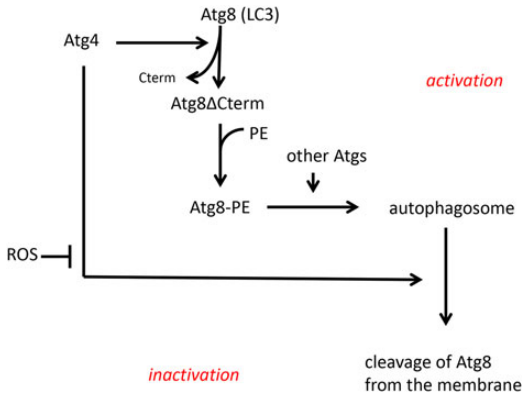

Fig. 2 The first steps of autophagy. The autophagy pathway proceeds through a series of stages, including nucleation of the autophagic vesicle, elongation and closure of the autophagosome membrane to envelop cytoplasmic constituents, docking of the autophagosome with lysosomes and degradation of the material inside the autophagosome. For details, see Ref. 43. This picture shows an essential step in the process of elongation, i.e. lipidation of Atg8-like proteins, such as LC3, and targeting of these lipidated proteins to the autophagosomal membrane. The delipidation of these proteins, also by Atg4, is inhibited by ROS, the source of which can vary with the cell involved and the cause of autophagy induction. In case of disturbed ROS production, as in CGD macrophages, the delipidation will be increased and autophagosome formation will be decreased.

are probably degraded. However, Atg4 can also delipidate Atg8 and release it from the membrane for recycling. Thus, Atg4 must be tightly regulated to ensure sufficient Atg8 lipidation for autophagosome maturation. This regulation of Atg4 has been extensively investigated, and it was found that ROS are involved in this process.[33] More precisely, the second, Atg8-delipidating step of Atg4 was found to be inhibited by ROS, in particular H_2O_2, whereas the first, lipidating reaction was not. In case of starvation, the mitochondria in a cell start to release H_2O_2, which then leads to more Atg8 lipidation and increased autophagy.

The pathways of anti-microbial defence and autophagy were found to be interconnected when it was discovered that autophagic proteins, in particular LC3 and beclin 1, also bind to phagosomes and promote their maturation (fusion with lysosomes) and the killing of the enclosed microbes.[34] Whether this should be called true autophagy or merely an alternative use of some autophagy proteins can be discussed, but I will use the term autophagy for both. This process is initiated not only by engagement of Toll-like receptors[34,35] but also by binding of the (opsonized) microorganisms to Fc receptors and dectin-1.[35] In mice, it was found that both macrophages and neutrophils utilize this pathway. Moreover, the binding of LC3 to phagosomes was strongly decreased when phagocytes from gp91^{phox} knock-out mice were used.[35] Thus, it appeared that in these cells H_2O_2 generated by the NADPH oxidase system is essential for phagosome maturation. Indeed, when COS cells transfected with Fc receptors and all components of the NADPH oxidase were used, again LC3 binding to the phagosomes was observed.[35] Apparently, no other anti-microbial signalling proteins are needed for this process. In addition, in human epithelial cells, the escape of *Salmonella* Typhimurium from phagosomes was found to be inhibited by the binding of LC3 to these vacuoles. The conclusion is that ROS have a dual role in anti-microbial defence: one direct toxic effect and one signalling role to promote autophagy and phagosome maturation.

Interestingly, also the well-known beneficial effect of interferon-gamma (IFNγ) by suppression of infections in CGD patients[16] may be related to the enhancement of autophagy by INFγ. This refers to so-called immunity-related GTPases (IRGs or p47 GTPases), whose expression is regulated by IFNγ. These IRGs are involved in IFNγ-induced autophagy and antimycobacterial activity in macrophages.[36,37]

These publications stimulated subsequent research on autophagy involvement in the antibacterial defence of CGD patients. Based on the mouse studies by Huang *et al.*,[35] it was to be expected that CGD neutrophils and monocytes would show a deficiency in LC3 lipidation and binding to phagosomes. Indeed, this is what De Luca *et al.*[38] found. However, these investigators also noticed that LPS-stimulated CGD monocytes release excessive amounts of IL-1β, a pro-inflammatory cytokine. In control monocytes but not in CGD monocytes, IL-1β release was enhanced when the autophagy process was inhibited by 3-methyladenine. This indicates that in CGD monocytes, the defect in autophagy is

causing the excessive IL-1β release. It also indicates that the deficient autophagy process in CGD patients not only causes a decreased bacterial killing process as a result of insufficient phagosome maturation, but also a hyperinflammatory state as a result of increased release of pro-inflammatory cytokines. How does this work? It is known for some time already that autophagy inhibits IL-1β transcription and processing of pro-IL-1β.[39–41] Pro-IL-1β processing takes place in the inflammasome, an intracellular structure that binds microbes and activates the enzyme caspase-1, which subsequently cleaves pro-IL-1β into active IL-1β, which is then released.[42] In addition, pro-IL-18 is cleaved by caspase-1 into active IL-18, another pro-inflammatory cytokine. Autophagy limits inflammasome activity by ubiquitination of the inflammasome component ASC and of bacterial components in the inflammasome, which then bind to the autophagic adaptor protein p62.[41] Subsequently, p62 binds LC3, which is then lipidated, and the whole complex is destructed in autophagosomes (Fig. 3). On the other hand, inflammasome formation also triggers activation of the small G protein RalB, which promotes autophagosome formation.[41] Moreover, increasing autophagy with rapamycin induces the degradation of pro-IL-1β in proteasomes.[41] Conversely, the work of de Luca *et al.*[38] indicated that limiting IL-1β release had a positive effect on LC3 recruitment. It seems, therefore, that autophagy and inflammasome activity are controlling each other's activity: IL-1β controls the rate of autophagy and autophagy controls the rate of IL-1β release.

The link between the defect in ROS production and the occurrence of inflammatory bowel disease in CGD may lie in the Paneth cells, specialized intestinal epithelial cells. Paneth cells function as a barrier

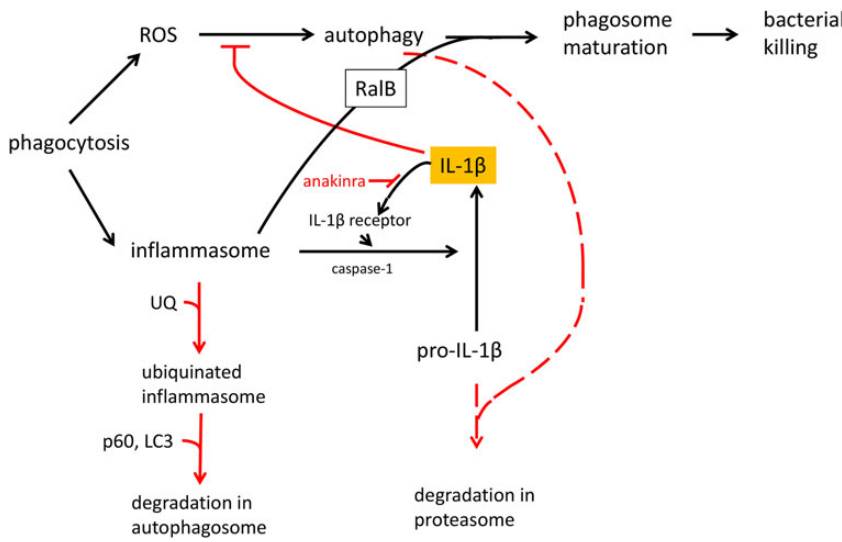

Fig. 3 Reciprocal effects of autophagy on inflammasome activity. During phagocytosis and when microbes have escaped into the cytoplasm, binding of their constituents to TLR receptors will induce inflammasome formation. This leads to IL-1β formation from pro-IL-1β. IL-1β is excreted from the cells but can bind to the IL-1β receptor and in this way accelerates the production of IL-1β. This binding to the IL-1β receptor is inhibited by anakinra, which thus limits IL-1β production. Since anakinra also limits LC3 lipidation, IL-1β supposedly limits autophagy. On the other hand, inflammasome formation also triggers activation of the small G protein RalB, which promotes autophagosome formation.[41] Moreover, autophagosome formation is connected with destruction of ubiquitinated inflammasomes and their contents by binding of the autophagic adaptor p62, which then binds LC3, followed by destruction in autophagosomes. Autophagy also limits IL-1β production by enhancing the degradation of pro-IL-1β in proteasomes.

to bacterial invasion, in part by secreting granule contents that contain antimicrobial peptides, and as regulators of intestinal inflammation. The autophagy protein Atg16L1 in mice regulates cytokine expression in these cells. Defects in Atg16L1 lead to enhanced IL-1β and IL-18 secretion by macrophages of mice challenged with dextran sulfate and contribute to the pathogenesis of Crohn's Disease.[43] Possibly, autophagy proteins are involved in proper handling of intestinal microbes by Paneth cells. It is thus conceivable that a defect in initiating autophagy, as in CGD, may lead to susceptibility to gut inflammation.

Encouraged by the beneficial treatment of patients with Familial Mediterranean Fever and other autoinflammatory diseases with the IL-1β receptor antagonist anakinra,[44] this drug has also been applied in CGD.[38] *In vitro* anakinra decreased inflammasome activation (indicating a feed-forward loop of IL-1β in promoting its own generation) and increased autophagy (indicating the above-mentioned inhibiting effect of IL-1β on autophagy). *In vivo*, anakinra prevented weight loss and histological pathology in p47 knock-out mice (but not normal mice) suffering from colitis induced by 2,4,6-trinitrobenzene sulfonic acid, indicating both the involvement of the NADPH oxidase activity and autophagy in colitis induction and also the role of IL-1β in this process. In another mouse model, *Aspergillus* conidia were given intranasally.[38] P47 knock-out mice had lower survival and more living aspergillus and neutrophils in the lung than normal mice. Anakinra prevented death, reduced fungal growth, decreased neutrophil influx, IL-1β and IL-17 levels in the lung and increased IFNγ and IL-10 levels in the lung. Two CGD patients were treated during 3 months with anakinra; both showed immediate and persistent improvement of their colitis symptoms.[38] In contrast, five other CGD patients with colitis, also treated for 3 months with anakinra, did not show lasting improvement.[45] Similar lack of success has been noted elsewhere (T.W. Kuijpers, personal communication). It remains to be seen, therefore, whether excessive IL-1β production is the real cause of gastrointestinal tract inflammation in CGD patients.

Another possible cause of these problems may be localized in the goblet cells of the colon. Goblet cells secrete mucus, which forms a layer that covers the intestinal epithelium and constitutes a barrier against intestinal microbes. Patel *et al.* have found that the autophagy protein LC3 in murine goblet cells localized to intracellular vesicles that were consistent with a fusion of autophagosomes and endocytic vesicles.[46] In addition, these fusion vesicles also contained the NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ (other subunits were not tested), which co-localized with LC3. Colonic epithelial cells from $p22^{phox}$- or $p47^{phox}$-deficient mice produced less ROS and showed more mucin accumulation than did wild-type cells. This indicates that ROS is required for mucin secretion. In the NADPH oxidase-deficient epithelial cells, mucin secretion was restored by addition of hydrogen peroxide, a process that involved calcium ion liberation in the cytoplasm of the cells. Thus, it seems plausible that both autophagy and ROS formation are needed for proper functioning of goblet cells. Whether this also implies that CGD patients may have dysfunctioning goblet cells, leading to increased susceptibility to gut inflammation, needs further study. Strangely, Patel *et al.*[46] did not use the well-known $gp91^{phox}$ knock-out mouse for their studies, nor do they mention the possible link of their results with gut inflammation in CGD.

Taken together, it is possible that the colitis manifestations seen in many CGD patients are caused by insufficient autophagy and excessive IL-1β generation or by goblet cell dysfunctioning. Dhillon *et al.*,[47] reasoning from the other side of this coin, investigated 112 patients with very early onset inflammatory bowel disease and found 11 different hypomorphic SNPs in components of the NADPH oxidase. IL-1β levels in these patients were not determined. Thus, the connection between these phenomena seems clear, but the exact causal relationship not yet.

T-cell activation

Another possible cause of the hyperinflammatory state of CGD patients is a defect in the regulation of T-lymphocyte activity. $P47^{phox}$ knock-out mice and rats develop more severe arthritis than wild-type animals when challenged with collagen-specific

T cells.[48] Transgenic mice that received normal p47^{phox}-expressing macrophages via adoptive transfer restored arthritis resistance to the level of that of wild-type mice in the T-cell-dependent collagen-induced model but not in a T-cell-independent anti-collagen antibody-induced arthritis model. *In vitro* investigations showed that two parameters of T-cell activation—proliferation and IL-2 production—are inhibited by macrophage ROS production. Recent experiments in our own laboratory have shown that human neutrophils display a similar ROS-dependent inhibitory action on human T-cell proliferation (Ida Hiemstra *et al.*, unpublished results).

Indications of ROS involvement in preventing arthritis were also obtained from positional identification of *Ncf1* (the gene encoding p47^{phox} in rodents) as a gene that regulates arthritis severity in rats.[49] A naturally occurring polymorphism in *Ncf1* causing reduced NADPH oxidase activity was found to be associated with more severe symptoms of pristane-induced arthritis. This arthritogenic effect originated in the immune-priming phase of the disease and could be prevented by intradermal injection of the NADPH oxidase-enhancing agent phytol.[49] Also in mice, the *Ncf1* gene was found to be involved in ameliorating the effects of collagen-induced arthritis.[50] Here too, increased activity of autoreactive T cells were found in *Ncf1*-mutated mice. Taken together, these observations indicate that macrophage-derived ROS are involved in inhibiting autoreactive T-cell responses.

Extending these observations to the human situation, this suggests that the arthritis seen in some CGD patients might be due to insufficient control of T-cell responses by NADPH-oxidase-generated ROS. Further research in this area is needed before definite conclusions can be drawn.

Other causes of hyperinflammation

ROS may not only restrict T-cell responses but also innate immune responses. In p47^{phox} knock-out mice, it was found that intratracheal challenge with zymosan or LPS caused exaggerated lung inflammation, augmented NF-κB activation and elevated release of pro-inflammatory cytokines (TNFα, IL-17 and G-CSF).[51] Transplantation of these mice with bone marrow from wild-type animals restored the normal lung inflammatory response. In macrophages from the p47$^{phox\ -/-}$ mice, zymosan and LPS failed to activate Nrf2, a redox-sensitive transcription factor involved in the generation of anti-oxidant proteins and led to enhanced NF-κB activation. In peripheral blood mononuclear cells (PBMCs) from CGD patients, zymosan completely failed to activate Nrf2 and induced augmented activation of NF-κB. Failure to downregulate NF-κB activation likely contributes to the exaggerated inflammatory response in CGD.

Conclusions

1. CGD is a rare but serious disease in which patients suffer from recurrent bacterial and fungal infections due to a deficiency in the leukocyte NADPH oxidase enzyme.
2. In addition, CGD patients also demonstrate a hyperinflammatory condition, leading to granulomas, colitis, non-infectious arthritis and other auto-immune diseases.
3. The origin of the hyperinflammation may be 2-fold: deficient autophagic antimicrobial defence, leading to excessive IL-1β release, and deficient regulation of autoreactive T cells.
4. For the clinician, it is important to correctly recognize the symptoms of CGD, to adequately treat the infections, to have the diagnosis confirmed by cellular and genetic evidence, to be aware that residual NADPH oxidase activity predicts a better prognosis, to choose the right moment for bone marrow transplantation and to give adequate genetic advice to the family.
5. For the laboratory investigator, it is important to correctly measure NADPH oxidase activity in the neutrophils of the patients (especially weak activity is important). In general, the DHR assay suffices, but one should be aware that myeloperoxidase deficiency can lead to a false-positive diagnosis of CGD. Correct genetic analysis should include all possible CGD genes, also *NCF1, NCF4, G6PD and Rac2*. For *NCF1*, it may be necessary to analyze the cDNA.

Areas timely for developing research

1. Choice of patients and time of treatment with bone marrow transplantation.
2. Development of safe and effective gene therapy.
3. Better definition of the origin(s) of the hyperinflammatory state.
4. The effects of SNPs in CGD genes on clinical expression of the disease.

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Conflict of interest statement

The authors have no potential conflicts of interest.

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